AFLP Analysis of Opium Poppy

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ABSTRACT

Amplified restriction fragment length polymorphic (AFLP) DNA analysis was performed on leaf samples of 40 accessions of opium poppy (*Papaver somniferum* L.) and two other control genera (*Papaver bracteatum* Lindley and *Papaver setigerum* DC.) from a commercial breeding collection held in Tasmania, Australia. A similarity dendrogram was produced on the basis of the analysis of all AFLP bands that ranged between 66 and 367 base pairs (bp) seen on an autoradiogram from denaturing polyacrylamide sequencing electrophoretic gels with three different primer pairs. It was necessary to combine the analysis of all three primer pairs into a single dendrogram to ensure that replicate analyses of related populations were grouped together. It was necessary to use more than one primer pair to resolve clearly the genetic relationship between the closely related plants described in this study; however, even a single primer pair could easily distinguish between populations of different poppy species. The application of AFLP DNA analysis for cultivar identification represents an efficient, reliable procedure for identifying opium poppy breeding lines in a definitive manner. This procedure required only a small amount of leaf material for analysis and the degree of the genetic relatedness was performed on autoradiographic banding patterns with commercially available software. These data were used to generate a similarity dendrogram depicting the predicted genetic relationship of the opium poppy cultivars. Opium poppy accessions sharing common parental lines could be distinguished unambiguously from poppy accessions of more distant genetic background. The banding patterns were reproducible, consistent within a genotype, and the DNA polymorphisms were frequent enough to be useful in characterizing genetic diversity in even closely related breeding lines.

The plant species *Papaver somniferum* (poppy) is grown commercially in several countries in secure environments to produce morphine for the world's pharmaceutical industries. The Australian island state of Tasmania is a major supplier of morphine for these industries. Commercial poppy crops have been grown in Tasmania since the 1970s with other major suppliers including India, Turkey and selected countries in Europe. To remain competitive with other international poppy industries, plant breeding programs in Tasmania have steadily increased endogenous morphine concentrations in poppy straw by conventional breeding methods. Although strides are being made in manipulating alkaloid levels through traditional breeding programs, the genetic origin and diversity of many of the accessions in the breeding collection is not fully known. This study was initiated to evaluate the genetic diversity of the breeding populations currently in use in Tasmania to provide information on those lines with the greatest genetic heterogeneity.

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There are several different DNA analysis procedures that have been used to identify and characterize plant cultivars to determine genetic diversity. Each procedure has it own requirements, sensitivity, and reliability. AFLP is the one of the newer DNA analysis procedures, which combines assay flexibility with a high degree of sensitivity and reproducibility to yield significantly more information about the plant genome under study than older techniques (Lin et al., 1996). Studies of genetic diversity based on AFLP DNA analysis have been applied to bacterial collections (Keim et al., 1997), fungal collections (O'Neill et al., 1997), nematode populations (Semblat et al., 1998), and accessions from several plant genera including *Brassica*, potato (*Solanum tuberosum* L.), soybean [*Glycine max* (L.) Merr.], barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), marijuana (*Cannabis sativa* L.), and lupines (*Lupinus* ssp.) (Brewer et al., 1999; Brien et al., 1999; Griffiths et al., 1999; Saunders et al., 1998; Travis et al., 1996).

The AFLP procedure is based on the digestion of genomic DNA by endonuclease restriction enzymes followed by the ligation of adapters. The adapter sequence, restriction site, plus additional selected nucleotides are used as priming sites for PCR amplification. Increased selectivity to differentiate between closely related samples can be achieved by modifying the user selected nucleotides.

To test the ability of AFLP markers to discriminate between the gene pools within *P. somniferum* and to test the potential application of genetic markers to identify poppy cultivars, DNA from 41 poppy accessions including two related species were subjected to AFLP analysis.

MATERIALS AND METHODS

Plant Material

A selection of accessions of *P. somniferum* were chosen from diverse poppy growing areas worldwide for this study (Table 1). In addition to samples of current and former commercial lines, representatives from land races and agro-botanical collections were also included. The hard seeded nondomesticated types from within the species *P. somniferum* were also examined, both as an accession from a gene bank and as volunteer outcrosses found in commercial poppy fields. Accessions were chosen to represent the extensive genetic diversity that can be found in *P. somniferum*. Individuals from each of two related species, *P. bracteatum* and *P. somniferum* ssp. *setigerum*, were also included to compare and contrast with genetic variation within *P. somniferum*. The *P. somniferum* ssp. *setigerum* accession used in this study was found in a commercial poppy field in Tasmania, Australia.

DNA Extraction

Fresh leaf tissue was taken from 60 individual poppy plants shown in Table 1, and grown in the field or greenhouse under

Abbreviations: AFLP, amplified restriction fragment length polymorphism; bp, base pair; PCR, polymerase chain reaction.

normal cultural practices for northern Tasmania (Laughlin et al., 1998). Because the collection was derived from a hitherto uncharacterized genetic population, the degree of heterogeneity was unknown. For this reason, several samples were replicated to access the degree of heterogeneity that might be expected from the population at large. Approximately 2 g of leaf tissue for each sample were deribbed and ground in liquid nitrogen with a mortar and pestle. Genomic DNA was isolated by extracting the ground leaf material with 3 mL of extraction buffer [50 m*M* Tris-HCl buffer pH 8.0, 1% (w/v) hexadecyltrimethylammonium bromide (CTAB), 50 m*M* Na_2EDTA, and 0.7 *M* NaCl] containing freshly added 1% (v/v) β-mercaptoethanol. The homogenate was incubated with gentle shaking at 60°C for 1 h with inversion mixing at 20-min intervals. Phenolic compounds were removed from older leaf samples by adding 0.1 g of insoluble polyvinylpolypyrrolidone (PVPP) per tube and centrifuging the samples for 10 min at 2000 × *g*. The supernatant was collected, cooled for 10 min on ice, and extracted in an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The samples were centrifuged at 10 000 × *g* for 20 min at 4°C, the upper layer was removed with a wide bore pipette and the volume determined. To precipitate the DNA, 1/10 volume of cold 3 *M* sodium acetate (pH 8.0) and 3/4 volume of cold isopropanol was added and the sample mixed by careful inversion. The DNA precipitated at −20°C within 1 h of storage and was spooled out of a microfuge tube with a glass rod. The spooled DNA was washed twice with 200 μL of 70% (v/v) ethanol and allowed to air dry for 20 min. The DNA was resuspended in 300 μL of TE buffer (1 m*M* Na_2 EDTA, 10 m*M* Tris-HCl pH 8.0) and stored at 4°C.

AFLP Analysis

AFLP DNA analysis was performed with the AFLP Analysis System I kit (Life Technologies, Inc., Gaithersburg, MD) following manufacturer's instructions and as modified from a previously described procedure (Lin et al., 1996). Approximately 500 ng of genomic DNA was digested for 2 h at 37°C by 2 μL *Eco*RI/*Mse*I restriction enzyme solution [1.25 Units/μL of both *Eco*RI and *Mse*I, 10 m*M* Tris-HCl pH 7.5, 50 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.1 mg/mL bovine serum albumin (BSA), 50% (v/v) glycerol, 0.1% (v/v) Triton X-100] and mixed with 5 μL of 50 m*M* Tris-HCl pH 7.5 containing 50 m*M* Mg-acetate and 250 m*M* K-acetate. The solution was brought to a final volume of 25 μL with H_2O. The reaction was stopped by enzyme denaturation at 70°C for 15 min.

PCR adapters were ligated to the digested DNA with a 24-μL *Eco*RI and *Mse*I adapter/ligation solution containing 0.4 m*M* ATP, 10 m*M* Tris-HCl (pH 7.5), 10 m*M* Mg-acetate, 50 m*M* K-acetate, and 1 μL T4 DNA ligase solution [1 Unit/μL in 10 m*M* Tris-HCl pH 7.5, 1 m*M* DTT, 50 m*M* KCl, and 50% (v/v) glycerol]. The reaction mixture was incubated at 20°C for 2 h and diluted 10-fold with TE buffer (10 m*M* Tris-HCl pH 8.0, 1 m*M* Na_2EDTA). Samples were PCR amplified with a Gene Amp 9700 PCR System (Perkin Elmer Applied Biosystems, Norwalk, CT) using a 20-cycle temperature profile at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. The reaction mixture contained 5 μL of the diluted DNA sample, 5 μL of PCR buffer (100 m*M* Tris-HCl pH 8.3, 15 m*M* $MgCl_2$, and 500 m*M* KCl), 1 unit of *Taq* DNA polymerase (Life Technologies, Inc., Gaithersburg, MD), and 40 μL of a mixture containing *Eco*RI + A and *Mse*I + C primers and dNTPs (Vos et al., 1995).

An aliquot of this PCR amplification was diluted 50-fold in TE buffer and used as template for a second, selective, ^{32}P-labeling PCR reaction. The reaction mixture contained 0.18 μL of *Eco*RI + 3 primer solution (27.8 ng/μL), 0.1 μL kinase buffer (350 m*M* Tris-HCl pH 7.6, 50 m*M* $MgCl_2$, 500 m*M* KCl, and 5 m*M* β-mercaptoethanol), 0.2 μL [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, Inc., Arlington Heights, IL) containing 2 μCi, and 0.2 μL T4 polynucleotide kinase [10 units/μL in 50 m*M* Tris-HCl pH 7.6, 25 m*M* KCl, 1 m*M* β-mercaptoethanol, 0.1 μ*M* ATP, 50%(v/v) glycerol]. The reaction proceeded at 37°C for 1 h and was stopped by enzyme inactivation at 70°C for 10 min.

Table 1. Origin of poppy accessions used for AFLP DNA analysis.

Poppy accession	Origin	Replications
UNL 110	Australia	3
Tascv-1	Australia	3
Tascv-2	Australia	3
Tascv-3	Australia	3
Tascv-4	Australia	2
Tascv-5	Australia	2
Tascv-6	Australia	2
Tascv-7	Australia	2
Volunteer-1	Australia	1
Volunteer-2	Australia	1
Afghan 96V0163-1-1	Afghanistan	2
Afghan 96V0169	Afghanistan	2
NR96134	Czechoslovakia	2
NR96137	Holland	2
NR96133	France	3
SC Morphinmak DDR-1	Hungary	2
Goluboj Jubilejnyi PAP250	Russia	1
Madurovics	Hungary	1
Rustica "O"	Spain	1
PAP-098	Romania	1
PAP-199	Romania	1
Sinjocaftast P360 PAP-327	Bulgaria	1
Sinjocaftjast S188 PAP-328	Bulgaria	1
PAP-498	Romania	1
PAP-501	Romania	1
PAP-519	Romania	1
PAP-520	Romania	1
PAP-710	Bulgaria	1
PAP-711	Bulgaria	1
PAP-722	Romania	1
PAP-725	Romania	1
PAP-785	Romania	1
PAP-799	Romania	1
PAP-800	Romania	1
PAP-801	Romania	1
PAP-454	Russia	1
UNL-208	India	1
UNL-204	India	1
UNL-139	Pakistan	1
Papaver bracteatum	Iran	1
P. somniferum (*setigerum*)	Australia	1

For selective PCR amplification, the primers were extended by two additional user selected nucleotides such that the reaction mixture consisted of three stock solutions, 5 μL of diluted preamplified sample, 5 μL of primer cocktail, and 10 μL of enzyme cocktail. The primer cocktail contained 0.5 μL of ^{32}P-labeled *Eco*RI + 3 primer and 4.5 μL of unlabeled *Mse*I + 3 primer (6.7 ng/μL) containing dNTPs. Four primer combinations containing the adapter plus 3 base primer pair sets were tested, *Eco*RI/*Mse*I ACT/CAG, AAC/CTT, AAC/CTC and ACT/CAT. The PCR enzyme cocktail contained 7.9 μL water, 2 μL PCR buffer, and 0.1 μL of *Taq* polymerase (0.5 Units). The PCR profile was 1 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by 12 cycles lowering the annealing temperature from 65°C to 56°C at −0.7°C steps for each cycle, and finally 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

After PCR amplification, 20 μL of formamide dye [98% (w/v) formamide, 10 m*M* Na_2EDTA, 100 mg/100 mL bromophenol blue, and 100 mg/100 mL xylene cyanol] was added, and the mixture was heated at 90°C for 3 min and stored at 4°C.

A 6% (w/v) polyacrylamide gel containing 19:1 acrylamide: bisacrylamide, 7.5 *M* urea, and 1× TBE buffer (0.1 *M* Tris-HCl, 0.09 *M* boric acid, 0.001 *M* EDTA) was used with 0.4-mm spacers and a sharkstooth comb, and the gel was preelectrophoresed at 50 W for 20 min. A 2-μL aliquot of the sample was electrophoresis at 50 W. The gel was dried and exposed overnight to X-ray film without an intensifying screen at −80°C.

Data Scoring and Analysis

The autoradiograms from each of the primer sets were scanned into a computer and scored for the presence of AFLP bands that could be clearly resolved in the general range of 60 to 360 bp. Molecular weights of the AFLP bands were determined by means of a 50-330 10-bp ladder (Life Technologies, Inc., Gaithersburg, MD) using DNA ProScore version A.36 software (DNA ProScan, Inc., Nashville, TN). The resulting binary data matrices for each primer pair were appended and subjected to cluster analysis using the software Numerical Taxonomy and Multivariate Analysis System (NTSYS, version 1.60, Applied Biostatistics Inc.; Rohlf, 1990). Clustering of samples was based on measures of Euclidean distance between the banding pattern, for all possible pairwise comparisons between DNA samples. A similarity dendrogram was produced from the cluster analysis software using unweighted pair-group method with arithmetic average (UPGMA).

RESULTS

A breeding collection of opium poppy used for commercial alkaloid production in Tasmania, Australia, was evaluated for genetic diversity by AFLP analysis. By separating electrophoretically the PCR amplified DNA fragments on a polyacrylamide gel, specific banding patterns indicative of individual accession were obtained. The banding patterns were reproducible within replicate experiments, consistent within a common accession, and show polymorphic bands that were frequent enough to characterize even closely related breeding lines. As little as 300 to 500 ng of genomic DNA could produce 60 to 90 PCR amplified DNA bands within the size range of 60 to 360 bp for each primer. Of the four primer pairs initially evaluated in this study for their ability to produce unique DNA banding patterns, three randomly selected primer pair combinations, *Eco*RI/*Mse*I ACT/CAG, AAC/CTT, and AAC/CTC, produced more than twice the number of polymorphic DNA bands as the ACT/CAT primer pair set. For this reason, the ACT/CAT primer pair was not used to generate DNA band patterns for the full set of accessions and was excluded from further study. A similarity data matrix for each primer pair was analyzed separately (data not shown) and in a combined matrix. An aggregate similarity dendrogram was produced from 246 bands of the three combined primer sets, *Eco*RI/*Mse*I ACT/CAG, AAC/CTT and AAC/CTC.

Figure 1 shows a typical autoradiogram using the single primer pair AAC/CTT combination on a series of 30 opium poppy samples. AFLP data derived from all samples of the three primer were combined (Table 2). Among the 246 DNA bands that were scored across all accessions, 22 DNA bands were conserved throughout all samples tested, including two related species *P. bracteatum* and *P. somniferum* ssp. *setigerum*. The remaining 224 DNA bands (91%) were polymorphic for at least one of the accessions in the population being examined. Since the *P. bracteatum* and *P. somniferum* ssp. *setigerum* samples were included as control outliers, a more revealing analysis of accessions exclusively from *P. somniferum* breeding lines are also shown in Table 2. As expected, the number of conserved bands within *P. somniferum* commercial accessions is much higher (60/216) than across species (22/246). Thus the percentage of polymorphism is significantly lower (73%) within these commercial breeding lines, reflecting the narrower genetic diversity of the population. Within the three primer pairs tested, the *Eco*RI-AAC/*Mse*I-CTC primer amplified the most PCR DNA bands but had the lowest polymorphisms.

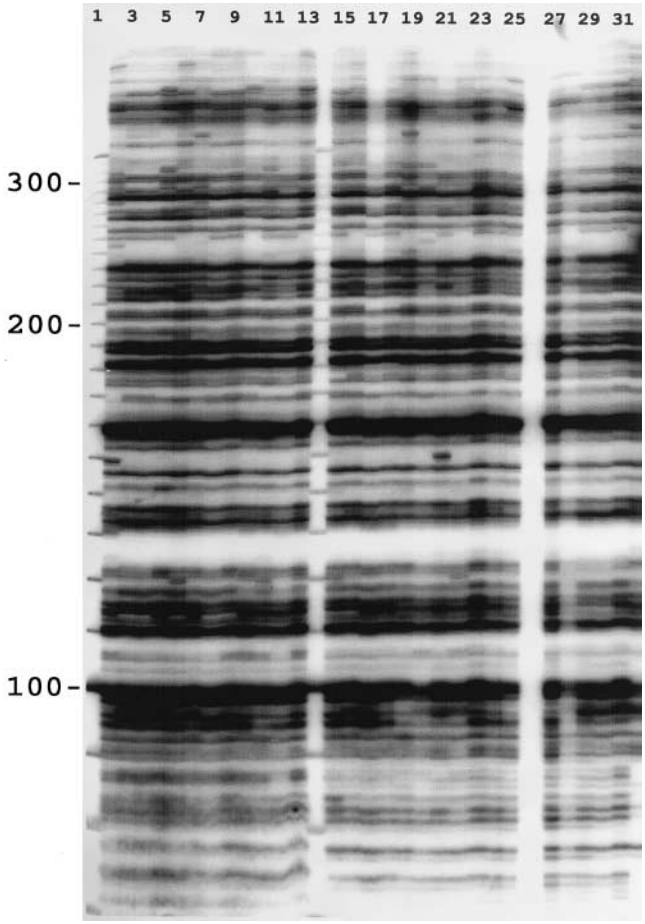

Fig. 1. An autoradiogram of the AFLP bands from the primer pair *Eco*RI-AAC/*Mse*I-CTT. Lanes 1 and 14 are 10-bp marker bands from 70 to 330 bp. All other lanes represent randomly selected poppy accessions within the pool of samples listed in Table 1. Note conserved bands (100, 189, 191, etc.) shared by all samples at numerous fragment sizes and polymorphic bands (149, 170, 250 etc.).

The dendrogram produced by the UPGMA cluster analysis of the pooled AFLP data from three primer pairs is presented in Fig. 2. Replicate samples from within poppy accessions tended to be clustered very closely together, with similarity indices near 1.0. In the case of the replicate samples NR96137 (from Holland);

Table 2. Number of AFLP bands scored by means of three primer sets consisting of *Eco*RI-AAC/*Mse*I-CTC, *E*-AAC/*M*-CTT and *E*-ACT/*M*-CAG.

AFLP primer set	*E*-AAC, *M*-CTC	*E*-AAC, *M*-CTT	*E*-ACT, *M*-CAG	Combined primers
Band size range scored	**66–353**	**75–327**	**81–367**	**66–367**
P. somniferum				
Total bands	**94**	**61**	**61**	**216**
Conserved bands	**31**	**15**	**14**	**60**
Polymorphic bands	**63**	**46**	**47**	**156**
% polymorphism	**67%**	**75%**	**77%**	**73%**
All accessions				
Total bands	**101**	**64**	**81**	**246**
Conserved bands	**8**	**5**	**9**	**22**
Polymorphic bands	**93**	**59**	**72**	**224**
% polymorphism	**92%**	**92%**	**89%**	**91%**

NR96133 (France); Tascv-3, Tascv-4, and Tascv-5 accessions (Australian cultivars); and the 96V0163 (Afghan) inbred lines, the three primer combinations showed AFLP banding patterns that were nearly identical. Only two sets of samples from replicate accessions failed to cluster above 97% identity, N96134 (Czechoslovakia) clustered at 92% identity, and the one accession of the three replicates of Tascv-1 (Australia) clustered at 94% identity. This minor variation may reflect scoring errors for the banding patterns or it may reflect natural heterogeneity within the population being sampled.

In view the reliability of the DNA banding patterns within the accessions, it is reasonable to allow relationships between samples to be deduced from the DNA analysis. Two accessions (Volunteer 1 and 2) collected in the same area within an Australian poppy breeding field were clustered closely together, and only slightly less closely with Rustica "O" (Fig. 2), to which they were phenotypically very similar. Rustica had been grown for several years previously in the area where the volunteer accessions were found, and it is likely that the volunteers were the result of an outcrossing between Rustica and Australian poppy cultivars.

Replicate Australian cultivars clustered together but were distributed in separate groups throughout the dendrogram intermixed with more loosely clustered Western European cultivars. This reflects the diverse nature of the accessions imported into Australia at different times from different sources. The Romanian accessions clustered in a separate group from the Bulgarian, Afghan, and Pakistani accessions, each of which in turn formed separate clusters. This result depicts the historical origins and diversity of opium poppy throughout these geographic regions of the world. Madurovics, a poppy variety noted for its short flowering cycle, did not cluster closely with any accession (Andrasfalvy, 1968) and reflects the unique character of this line.

Of the two control outlier poppy plants tested, *P. bracteatum* did not cluster closely to any accession, while *P. somniferum* ssp. *setigerum* appeared closer to one clad of *somniferum* accessions than the other poppy samples. These findings are in accordance with the accepted view that *setigerum* is closely related to *somniferum*, some even classifying it to be a subspecies (Hammer, 1981; Tetenyi, 1995). *Papaver somniferum* has much greater morphological similarity with *P. somniferum* ssp. *setigerum* than with *P. bracteatum*. *Papaver bracteatum* is a perennial shrub that is noted for its accumulation of the alkaloid thebaine, and unlike *P. somniferum* does not possess the biosynthetic capacity to demethylate thebaine to allow the accumulation of morphine.

DISCUSSION

The number and pattern of conserved and polymorphic bands within a group of related cultivars is a function of the genetic variability within the plant collection being analyzed. It is also partially under the control of the selection of primers that were used to prepare the DNA for PCR amplification. Initially, a double digestion of genomic DNA was performed with *Eco*RI and *Mse*I endonuclease restriction enzymes. Specific sequenced primer adapters were ligated onto both ends of the restricted genomic DNA. The adapter sequence, the sequence of the restricted genomic DNA, and three additional user selected nucleotides are used as primer binding sites for PCR amplification. The specificity of amplification is determined by the three additional selected nucleotides that correspond to the first base pairs of the genomic DNA beyond the restriction site. Changing these three base pairs in the primer, or using less than 3 selective base pairs to reduce the selectivity of the primer set can be used as a dynamic tool to examine the genetic diversity within specific plant populations. While the three primer pair combinations used in this study, *E*-ACT/*M*-CAG, *E*-AAC/*M*-CTT, and *E*-AAC/*M*-CTC, clearly differentiated the cultivars tested, subtle differences in the similarity dendrogram may have occurred with different primer pair combinations. By comparing the individual dendrograms from each primer pair tested with the composite dendrogram of all three primer pairs, a more comprehensive analysis was achieved. Although these primer sets were chosen at random, we assumed that not all primer pairs provide the same degree of genetic information about the genome under study. For example, each of the randomly chosen primer pairs listed above produced more than twice the number of polymorphic DNA bands as a fourth randomly selected primer combination *E*-ACT/*M*-CAT. For this reason, only these first three primer pairs were subsequently used in the data analysis.

There is some question as to the validity of using similarity dendrograms in deducing evolutionary development within germplasm pools. Similarity dendrograms produced via the unweighted pair-group method with arithmetic averaging for cluster analysis are an adequate tool for depicting genetic relatedness among accessions generally (O'Neill et al., 1997). However, the elucidation of evolutionary relationships on the basis of genetic fingerprinting may require assumptions that genetic change is progressing at a constant rate over time and that any changes made are independent genetic events (Weir, 1996). In the case of cultivated crops, which have been under a selective breeding program for many generations that focused on specific phenotypic or biochemical traits, these assumptions are generally not

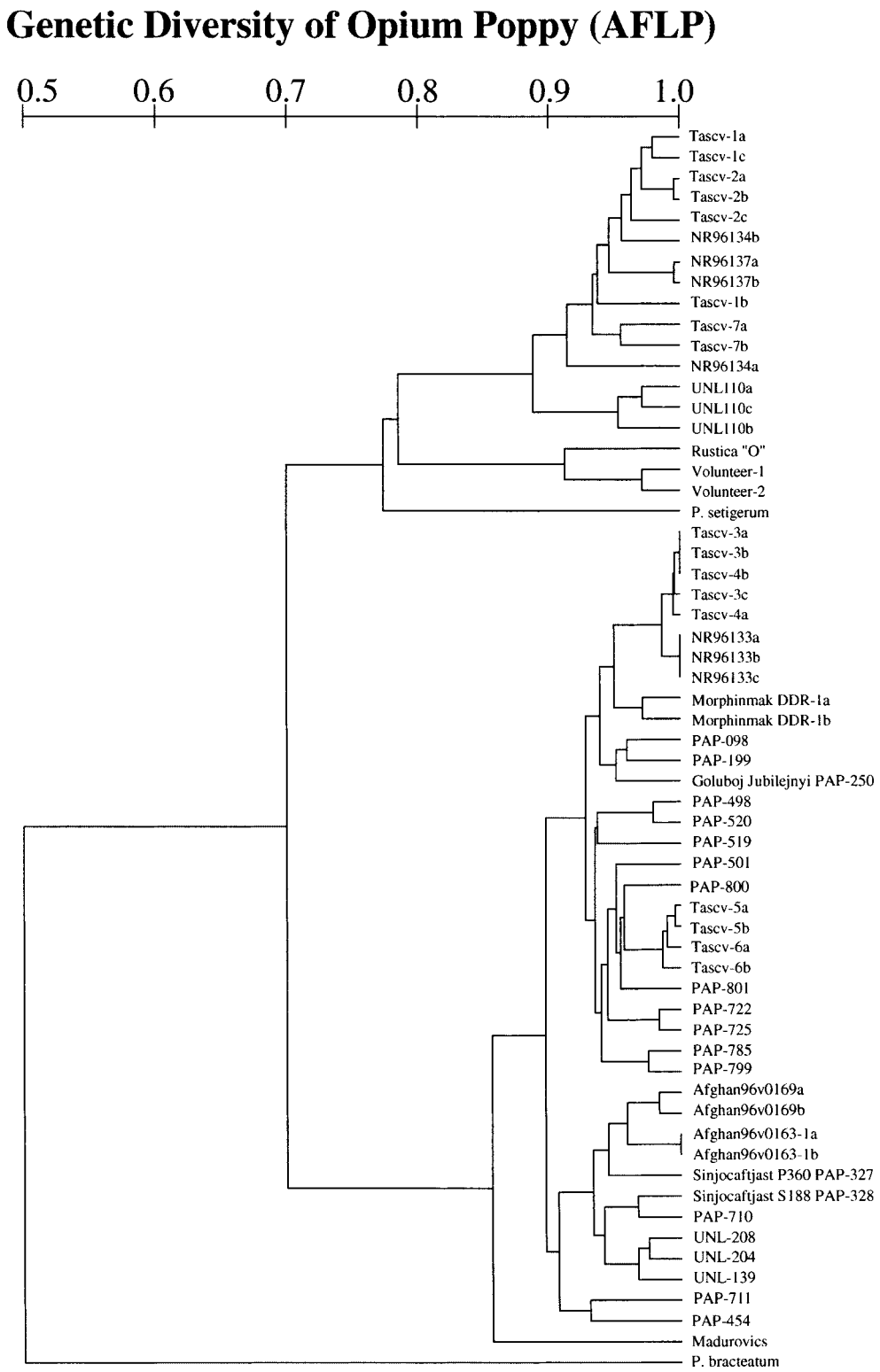

Fig. 2. A similarity dendrogram produced by UPGMA analysis of AFLP data derived from three primer pairs, *Eco*RI- ACT/*Mse*I-CAG, *E*-AAC/*M*-CTT and *E*-AAC/*M*-CTC. The origin of each of the poppy accession is listed in Table 1. Replicate samples are designated as (a,b,c).

met. In the poppy collections used in this study, there has been considerable intermixing of gene pools from one part of the world to another both in historical times (Tetenyi, 1997) and in modern breeding programs. Thus, it is not surprising that Australian poppy cultivars, which have arisen from a wide variety of genetic backgrounds, have not clustered together into a single unit on the basis of geography, but into groups, reflecting the use of different parents in their genetic development.

AFLP DNA analysis has been shown to be a reliable

technique for exploring genetic diversity in plant populations (Hill et al., 1996; Paul et al., 1997). We have shown that the procedure can not only distinguish between related genetic species, such as *P. bracteatum* and *P. setigerum*, but also differentiate cultivars of *P. somniferum*. These data are useful for defining a cultivar type and predicting morphological parameters such as the alkaloid content of a mature plant, while at an early seedling stage. This aids in the selection and identification of high yielding chemical profiles of poppy plants in breeding programs.

The use of DNA cultivar typing in opium poppy samples is not only useful for cultivar identification and breeding programs, but has potential interest to individuals who would like to identify the geographic sources of poppy plants taken from collections around the world because of the unique chemical nature of the plants. AFLP profiles can help to identify historical and current redistributions of poppy germplasm. In our study, cultivars of opium poppy sharing a common genetic heritage could be distinguished easily from lines of a more distant genetic background. Although these accessions have been used in our breeding programs for many years and the site of collection of the germplasm recorded, the genetic relatedness of these lines were unknown because of the widespread historical distribution of this species. Analysis of the genetic relatedness performed on autoradiographic banding patterns by commercially available software allowed us to generate a similarity dendrogram depicting the predicted phylogenic relationship of the opium poppy cultivars. This type of analysis allows for unambiguous cultivar identification with limited fresh plant material.

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